

AMENDMENTS TO THE SPECIFICATION

Please replace paragraph [0007] with the following amended paragraph:

[0007] **Figure 1:** Comparison of the protein sequences of the reductoisomerases of *M. grisea* (SEQ ID NO:1) and *N. crassa* (SEQ ID NO:3) and of the yeast *S. cerevisiae* (SEQ ID NO:2). Sequence alignment using the CLUSTAL W(1.4)TM software. Symbols: ":" marks similar amino acids; "*" marks identical amino acids.

Please replace paragraph [0061] with the following amended paragraph:

[0061] An internal fragment of the *M. grisea* *ILV5* gene was amplified by PCR from the genomic DNA of this fungus using pairs of degenerate primers corresponding to protein domains which are conserved between fungal reductoisomerases. The PCR product obtained was then cloned into the plasmid pGEM[®]-T-Easy (Promega), sequenced, and amplified by PCR with a new pair of primers. The latter PCR product was used as a homologous probe for screening an *M. grisea* cosmid DNA library. The sequence of the *M. grisea* *ILV5* gene was then produced using one of the positive clones and oligonucleotides derived from the sequence of the PCR product already obtained.

Please replace paragraph [0064] and the preceding sentence with the following amended paragraph:

1.1.3. Cloning of a PCR-Amplified Internal Fragment of the *M. grisea* *ILV5* Gene, in the Plasmid pGEM[®]-T-easy

[0064] The internal fragment of the *M. grisea* *ILV5* gene was amplified by PCR from the *M. grisea* genomic DNA, with the pair of primers (1-2), at a primer hybridization temperature of 50°C for 3 cycles, and then 55°C for the other amplification cycles. This PCR product, of approximately 680 bp, is purified after separation by agarose gel electrophoresis and then cloned into the plasmid pGEM[®]-T-easy. The bacterial colonies obtained after transformation, and using a white/blue selection system (X-Gal), showed three different phenotypes: white, blue, and white with the center

of the colony being blue (called white/blue colonies). 30 colonies of various phenotypes were analyzed by PCR using the universal primers Sp6 and T7, which hybridize on either side of the cloning site of the plasmid pGEM®-T-easy. The 20 white colonies and the 10 white/blue colonies are positive. In fact, from these colonies, a DNA fragment was amplified at the expected size (810 bp), which corresponds to the size of the insert (680 bp) plus the distance separating the insert from each of the primers Sp6 and T7 (129 bp). Two clones of different phenotypes, white and white/blue, were then chosen in order to be sequenced. These are clones no. 4 (white) and no. 20 (white/blue).

Please replace paragraph [0065] with the following amended paragraph:

[0065] Comparison of the nucleotide sequences of the two clones no. 4 and no. 20 showed that they correspond to the same DNA fragment cloned in different orientations into the plasmid pGEM®-T-easy, which might explain their phenotypic difference (white and white/blue). The double-stranded nucleotide sequence of this cloned fragment was thus obtained. The homology of this nucleotide sequence with those encoding known proteins was sought using the Blastx BLASTX™ program from NCBI (National Center for Biotechnology Information). This program compares the translated sequences of the six reading frames of a nucleotide sequence with all the protein sequences contained in the databases. Significant homology between the nucleotide sequence of the cloned fragment and the protein sequence of the *N. crassa* reductoisomerase (error of e-102) was identified. The percentage amino acid identity within the region defined by the software for comparing these two sequences is 94%. The fragment cloned into the plasmid pGEM®-T-easy therefore corresponds to the internal fragment of the *M. grisea* *ILV5* gene. Although the sequence of the internal fragment of the *M. grisea* *ILV5* gene and that of the *N. crassa* *ILV5* gene exhibits strong homology, a difference exists at the center of the sequence of the *M. grisea* *ILV5* gene. This difference might correspond to the presence of an intron within the *M. grisea* sequence, since a 77 bp intron exists in *N. crassa* at this position. The position in the sequence of this intron was sought. A 5' splicing consensus motif was identified in the nucleotide sequence of the internal

fragment of the *M. grisea* *ILV5* gene, along with a 3' splicing consensus motif and a lariat sequence. The putative intron (86 bp) of the internal fragment of the *M. grisea* *ILV5* gene was therefore identified. Splicing of the intron of the sequence of the internal fragment of the *M. grisea* *ILV5* gene made it possible to obtain a "theoretical" cDNA fragment. The fragment of the protein sequence of the *M. grisea* reductoisomerase then deduced from this "theoretical" cDNA was compared with that of the *N. crassa* reductoisomerase, showing very strong identity between the primary sequences of these two enzymes (**Figure 1**).

Please replace paragraph [0066] with the following amended paragraph:

[0066] An internal fragment of the *M. grisea* *ILV5* gene was amplified by PCR, from clone no. 4, using the primers 13U and 549L defined on the basis of the sequence of the *ILV5* gene cloned into the plasmid pGEM®-T-easy. This fragment, after purification on agarose gel, was used as a matrix to prepare a labeled probe for the *ILV5* gene.

Please replace paragraph [0070] with the following amended paragraph:

[0070] The cDNA of the *M. grisea* *ILV5* gene was isolated by carrying out a PCR amplification from a cDNA library of the isolate P1.2 (RNA of mycelium cultured in complete medium) using the oligonucleotides defined on the basis of the *ILV5* gene sequence: oligonucleotides 22U and 1603L. Oligonucleotide 22U is located before the translation-initiating ATG and oligonucleotide 1603L is located 93 bp after the translation-terminating STOP codon. Two fragments are amplified with this pair of primers: a fragment less than 500 bp in size and a fragment amplified at the expected size, i.e. 1.6 kb. The fragment amplified at the expected size is purified after separation by agarose gel electrophoresis and cloned into the plasmid pGEM®-T-easy. The 24 bacterial clones obtained after transformation are analyzed by PCR using the pair of primers 22U and 1603L. These 24 clones possess the cDNA of the *M. grisea* *ILV5* gene, since a DNA fragment was in fact amplified at the expected size (1.6 kb). Clone no. 18 was chosen in order to be sequenced using the universal primers Sp6 and T7. Comparison of the nucleotide sequence of the cDNA of the *ILV5* gene allowed

us to determine the exact position of the introns. The three introns are located at the positions predicted by the comparison of the protein sequences of the *N. crassa* reductoisomerase and of the translations of the *M. grisea* *ILV5* gene. In *N. crassa*, the *ILV5* gene has 4 introns which are positioned differently and are different in length compared to the *M. grisea* *ILV5* gene.

Please replace paragraph [0098] with the following amended paragraph:

[0098] Overexpression of the “short” form (construct no. 1) and of the “long” form (construct no. 2) of the yeast reductoisomerase was induced in *E. coli* with IPTG. The bacterial strain BL21 pLysS, transformed with the plasmid pET 23-d containing the yeast *ILV5* gene without the region encoding the transit peptide, is cultured at 28°C with shaking in LB medium supplemented with carbenicillin (100 mg/l) and with chloramphenicol (30 mg/l) until a density equivalent to an OD₆₀₀ of approximately 0.6 is obtained. The IPTG is then added at a final concentration of 0.4 mM and the bacteria are left in culture at 28°C with shaking for approximately 15 hours. This bacterial culture is then centrifuged (30 minutes, 4500 rpm); the bacterial pellet is resuspended in 15 ml of buffer (10 mM KH₂-K₂PO₄ (pH 7.5), 1 mM EDTA, 1 mM DTT and protease inhibitors: 1 mM benzamidine HCl, 5 mM aminocaproic acid) and sonicated using a Vibra-cell VIBRA-CELL™ disruptor (Sonics and Materials, Danbury, CT, U.S.A) for 15 minutes, at power 4, 40% of the total lysis time. The cell extract is centrifuged (20 minutes, 15 000 rpm); the supernatant, containing the soluble proteins, is then conserved at -80°C.

Please replace paragraph [0100] with the following amended paragraph:

[0100] Purification of the short form of the reductoisomerase was therefore carried out in two steps using the soluble protein fraction; first, on an anion exchange column (Q-Sephadex Q-SEPHAROSE™), and then on a permeation column (Superdex 75 SUPERDEX 75™). The soluble protein extract (15.5 ml; 227.8 mg of proteins), which contains the yeast reductoisomerase (crude extract), is applied to an anion exchange column, HiLoad 16/10 Q-Sephadex Q-SEPHAROSE™ (Pharmacia), connected to a Pharmacia FPLC™ system, pre-equilibrated with

10 mM KH₂-K₂PO₄ buffer/1 mM EDTA/1 mM DTT. The enzyme is eluted with 78 ml of this same buffer (flow rate = 1 ml/min; fraction size = 3 ml). The chromatographic fractions containing the yeast reductoisomerase are concentrated to 1.6 ml by centrifugation at 5500 rpm in a macrosep-10 unit (filtron). This extract (27.7 mg) is then applied to a HiLoad 16/60 Superdex 75 column (Pharmacia) connected to a Pharmacia FPLC™ system, pre-equilibrated with 25 mM Hepes-KOH buffer. The enzyme is eluted with 58 ml of this same buffer (flow rate = 1 ml/min; fraction size = 1 ml). The chromatographic fractions containing the yeast reductoisomerase (18.99 mg) are concentrated to 9.7 mg/ml by centrifugation at 5500 rpm in a 10K ~~microsep~~ MICROSEPT™ (filtron) and conserved at -80°C.

Please replace paragraph [0101] with the following amended paragraph:

[0101] After injection of the soluble protein fraction (approximately 230 mg) onto the ~~Q-Sephadex~~ Q-SEPHAROSE™ column, the yeast reductoisomerase is diluted with 10 mM KH₂-K₂PO₄ buffer/1 mM EDTA/1 mM DTT. There is in fact no need to elute this enzyme through the action of an increasing concentration gradient of phosphate buffer since preliminary experiments have shown that this enzyme is not retained by the column. After this first purification step, approximately 30 mg of protein were recovered and the yield from the purification in terms of activity is 55% (**Table 6**).

Please replace paragraph [0102] with the following amended paragraph:

[0102] Analysis of the ~~Q-Sephadex~~ Q-SEPHAROSE™ fraction pool on acrylamide gel shows that, after this first purification step, the enzyme is virtually pure. The ~~Q-Sephadex~~ Q-SEPHAROSE™ fraction pool is injected onto the gel filtration column and the yeast reductoisomerase is eluted with 25 mM Hepes-KOH buffer. After this 2nd purification step, approximately 20 mg of pure protein are recovered; the final yield from the two steps for purifying the yeast reductoisomerase is approximately 34%.

Please replace paragraph [0117] with the following amended paragraph:

[0017] For irreversible inhibitors, the k_{-0} can be considered to be negligible, the k_0 is then calculated by virtue of equation (3) using the KaleidaGraph KALEIDAGRAPH™ program.

Please replace paragraph [0122] with the following amended paragraph:

[0122] Application, to gel filtration (Superdex 75 SUPERDEX 75™), of the pool of yeast reductoisomerase fractions obtained after the 1st purification step shows that the reductoisomerase is eluted in a single peak and that its molecular mass is estimated at 67 kDa. Now, the expected molecular mass of the monomeric form of this enzyme is approximately 40 kDa and 80 kDa for the dimeric form under nondenaturing conditions. The molecular mass which is intermediate between the monomeric and dimeric forms of the yeast reductoisomerase confirms the existence of an equilibrium between these two forms of the enzyme. Only a rapid dynamic equilibrium between these two forms could explain a single elution peak being obtained in gel filtration. Specifically, if this equilibrium was slow, two elution peaks would have been observed on exiting gel filtration; one would correspond to the monomeric form, and the other to the dimeric form of the enzyme.